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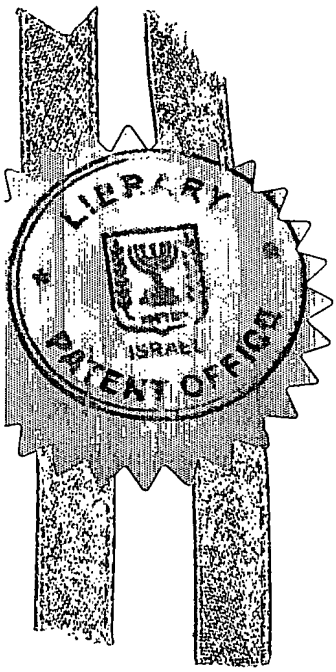

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I (Name and address of applicant, and in case of body corporate - place of incorporation)

METABOGAL LTD
P.O.Box 432
Kiryat Shemona 11013
Israel

מטבוגל בע"מ
ת.ד. 432
קרית שמונה 11013

Inventors:
Shaaltiel Yoseph
Bartfeld Daniel
Baum Gideon
Hshmueli Sharon

ממציאים:
שאלתיאל יוסף
ברטפלד דניאל
באום גדעון
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(English)

(באנגלית)

METHODS FOR EXPRESSION OF ENZYMATICALLY ACTIVE RECOMBINANT LYSOSOMAL ENZYMES IN TRANSGENIC PLANT ROOT CELLS AND VECTORS USED THEREBY

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המען למסירת מסמכים בישראל
Address for Service in Israel
לוצאטו את לוצאטו
LUZZATTO & LUZZATTO
ת.ד. 5352
P.O. Box 5352
באר שבע 84152
Beer-Sheva 84152

חתימת המבקש
Signature of Applicant
לוצאטו את לוצאטו
ב"כ המבקשת
LUZZATTO & LUZZATTO
ATTORNEYS FOR APPLICANTS

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METHODS FOR EXPRESSION OF ENZYMATICALLY ACTIVE RECOMBINANT LYSOSOMAL ENZYMES IN TRANSGENIC PLANT ROOT CELLS AND VECTORS USED THEREBY

Field of the Invention

The present invention relates to transformed host cells for the production of high mannose proteins. The invention further relates to vectors and methods for expression and production of enzymatically active high mannose lysosomal enzymes using transgenic plant root, particularly carrot cells. More particularly, the invention relates to host cells, particularly transgenic suspended carrot cells, vectors and methods for high yield expression and production of biologically active high mannose Glucocerebrosidase (GCD). The invention further provides for compositions and methods for the treatment of lysosomal storage diseases.

Background of the Invention

Gaucher's disease is the most prevalent lysosomal storage disorder. It is caused by a recessive genetic disorder (chromosome 1 q21-q31) resulting in deficiency of glucocerebrosidase, also known as glucosylceramidase, which is a membrane-bound lysosomal enzyme that catalyzes the hydrolysis of the glycosphingolipid glucocerebroside (glucosylceramide, GlcCer) to glucose and ceramide. Gaucher disease is caused by point mutations in the hGCD (human glucocerebrosidase) gene (GBA), which result in accumulation of GlcCer in the lysosomes of macrophages. The characteristic storage cells, called Gaucher cells, are found in liver, spleen and bone marrow. The associated clinical symptoms include severe hepatosplenomegaly, anemia, thrombocytopenia and skeletal deterioration.

There are three different types of Gaucher disease, each determined by the level of hGC activity. The major cells affected by the disease are the

macrophages, which are highly enlarged due to GlcCer accumulation, and are thus referred to as "Gaucher cells".

The identification of a defect in GCD as the primary cause of Gaucher's disease led to the development of enzyme replacement therapy as a therapeutic strategy for this disorder.

De Duve first suggested that replacement of the missing lysosomal enzyme with exogenous biologically active enzyme might be a viable approach to treatment of lysosomal storage diseases [Fed Proc. 23:1045 (1964)].

Since that time, various studies have suggested that enzyme replacement therapy may be beneficial for treating various lysosomal storage diseases. The best success has been shown with individuals with type I Gaucher disease, who were treated with exogenous enzyme (ß-glucocerebrosidase), prepared from placenta (Ceredase™) or, more recently, recombinantly (Cerezyme™).

Unmodified glucocerebrosidase derived from natural sources is a glycoprotein with four carbohydrate chains. This protein does not target the phagocytic cells in the body and is therefore of limited therapeutic value. In developing the current therapy for Gaucher's disease, the terminal sugars on the carbohydrate chains of glucocerebrosidase are sequentially removed by treatment with three different glycosidases. This glycosidase treatment results in a glycoprotein whose terminal sugars consist of mannose residues. Since phagocytes have mannose receptors that recognize glycoproteins and glycopeptides with oligosaccharide chains that terminate in mannose residues, the carbohydrate remodeling of glucocerebrosidase has improved

the targeting of the enzyme to these cells [Furbish *et al.*, Biochem. Biophys. Acta 673:425, (1981)].

As indicated herein, glycosylation plays a crucial role in hGCD activity, therefore deglycosylation of hGCD expressed in cell lines using either tunicamycin (*Sf9* cells) or point mutations abolishing all glycosylation sites (both *Sf9* and COS-1 cells), results in complete loss of enzymatic activity. In addition, hGCD expressed in *E. coli* was found to be inactive. Further research indicated the significance of the various glycosylation sites for protein activity. In addition to the role of glycosylation in the actual protein activity, the commercially produced enzyme contains glycan sequence modifications that facilitate specific drug delivery. The glycosylated proteins are remodeled following extraction to include only mannose containing glycan sequences.

The gene encoding human GCD was first sequenced in 1985 (Sorge et al., 1985, Proc. Nat. Acad. Sci. 2:7289-7293]. The protein consists of 497 amino acids derived from a 536-mer pro-peptide.

The enzyme contains 4 glycosylation sites and 22 lysines. The recombinantly produced enzyme (Cerezyme™) differs from the placental enzyme (Ceredase™) in position 495 where an arginine has been substituted with a histidine. Furthermore, the oligosaccharide composition differs between the recombinant and the placental GCD as the former has more fucose and N-acetyl-glucosamine residues while the latter retains one high mannose chain. As mentioned above, both types of GCDs are treated with three different glycosidases (neuraminidase, galactosidase, and P-N acetyl-glucosaminidase) to expose terminal mannoses, which enables targeting of phagocytic cells. A

pharmaceutical preparation comprising the recombinantly produced enzyme is described in US 5,549,892.

One drawback associated with existing lysosomal enzyme replacement therapy treatment is that the *in vivo* bioactivity of the enzyme is undesirably low, e.g. because of low uptake, reduced targeting to lysosomes of the specific cells where the substrate is accumulated, and a short functional *in vivo* half-life in the lysosomes.

Another major drawback of the existing GCD recombinant enzymes, is the fact that these are expensive form of therapy and place a heavy economic burden on health care systems. The high cost of these recombinant enzymes results from a complex purification protocol, and the relatively large amounts of the therapeutic required for existing treatments. There is therefore, an urgent need to reduce the cost of GCD so that this life saving therapy can be provided to all who require it more affordably.

Accordingly, there exists a need in the art for methods for providing sufficient quantities of biologically active lysosomal enzymes, and particularly, human GCD, to deficient cells. Additionally, there exists a need for host cells comprising new vector compositions that allow for efficient production of genes encoding lysosomal enzymes, such as GCD.

Since the hGCD glycosylation should be remodeled to generate high mannose structures in order to achieve active form, the expression of hGCD in plants could be of great value.

The basic biosynthesis pathway of high-mannose and complex N-linked glycans is highly conserved among all eukaryotes. The biosynthesis begins in the Endoplasmic Reticulum (ER) with the transfer of the glycan precursor from a dolichol lipid carrier to a specific Asn residue on the protein by the oligosaccharyl transferase. The precursor is subsequently modified in the ER by glycosidases I and II and a hypothetical mannosidase to yield the high mannose structures, similar to the process occurring in mammals.

Further modifications of the glycan sequence to complex and hybrid structures occurs in the Golgi. Such modifications include removal of one of the four mannose residues by α-mannosidase I, addition of an *N*-acetylglucosamine residue, removal of the two additional mannose residues by α-mannosidase II, addition of *N*-acetylglucosamine and optionally, at this stage, xylose and fucose residues may be added to yield plant specific N-linked glycans. After the transfer of xylose and fucose to the core, complex type N-glycans can be further processed via the addition of terminal fucose and galactose. Further modifications may take place during the glycoprotein transport.

Several approaches are currently utilized to control and tailor protein glycosylation in plants. Gross modifications, such as complete inhibition of glycosylation or the removal of glycosylation sites from the peptide chain is one strategy. However, this approach can result in structural defects. An additional approach involves knock-out and introduction of specific carbohydrate processing enzymes. The third approach tries to localize the expression to a specific compartment in the cell. For example, retaining the protein in the ER prevents plant specific modification from being carried out in the Golgi.

Since a high mannose structure of lysosomal enzymes is preferred, if secretion can be blocked and the protein can be maintained in the ER, naturally occurring high mannose structures will be obtained without the need for remodeling.

As indicated above, proteins transported via the endomembrane system first pass into the endoplasmic reticulum. The necessary transport signal for this step is represented by a signal sequence at the N-terminal end of the molecule, the so-called signal peptide. As soon as this signal peptide has fulfilled its function, which is to insert the precursor protein attached to it into the endoplasmic reticulum, it is split off proteolytically from the precursor protein. By virtue of its specific function, this type of signal peptide sequence has been conserved to a high degree during evolution in all living cells, irrespective of whether they are bacteria, yeasts, fungi, animals or plants.

Many plant proteins, which are inserted into the endoplasmic reticulum by virtue of the signal peptide do not reside in the ER, but are transported from the endoplasmic reticulum to the Golgi and continue trafficking from the Golgi to the vacuoles. One class of such sorting signals for this traffic resides are signals that reside on the C-terminal part of the precursor protein [Neuhaus and Rogers, (1998) Plant Mol. Biol. 38:127-144]. Proteins containing both an N-terminal signal peptide for insertion into the endoplasmic reticulum and a C-terminal vacuolar targeting signal are expected to contain complex glycans, which is attached to them in the Golgi [Lerouge et al., (1998) Plant Mol. Biol. 38:31-48]. The nature of such C-terminal sorting signals can vary very widely. US 6,054,637 describes peptide fragments obtained from the region of tobacco basic chitinase, which is a

vacuolar protein that act as vacuolar targeting peptides. An example for a vacuolar protein containing a C-terminal targeting signal and complex glycans is the phaseolin storage protein from bean seeds [Frigerio et al., (1998) Plant Cell 10:1031-1042; Frigerio et al., (2001) Plant Cell 13:1109-1126.].

The paradigm is that in all eukaryotic cells vacuolar proteins pass via the ER and the Golgi before sequestering in the vacuole as their final destination. Surprisingly, the transformed plant root cells of the present invention produced an unexpected high mannose GCD. Advantageously, this high mannose product was found to be biologically active and therefore no further steps were needed for its activation. Without being bound by theory, a new pathway that does not fit this paradigm was first discovered in wheat seeds where it is used to deliver storage proteins directly from the ER to the vacuole and is operating in parallel with the Golgi-mediated pathway [Vitale and Galili, (2001) Plant Physiol. 125:115-118]. Such a process is not unique to wheat seeds, and may represent a general mechanism of vacuole ontogeny in plants. As examples, similar processes were also shown to deliver from the ER to vacuoles, maize storage proteins expressed in transgenic tobacco seeds, storage proteins in pumpkin seeds, and likely also vacuolar membrane integral membrane proteins [Vitale and Galili, (2001) *ibid.*]

It is therefore an object of the present invention to provide host cells, vectors and methods for mass production of enzymatically active high mannose lysosomal enzymes.

This and other objects of the invention will become apparent as the description proceeds.

The present invention uses novel transformed host cells particularly designed for production of high mannose proteins. These cells are transformed with recombinant nucleic acid molecules comprising a vacuolar sequence for directing lysosomal proteins, preferably GCD, to the natural vacuole in carrot or other plant root cells. By using these particular host cells of the invention, the recombinant lysosomal enzymes were localized to a specific cell compartment such as the natural vacuole, and therefore, specific modifications carried out in the Golgi were avoided.

Thus, the present invention provides a novel, scaleable, cost-effective production and purification process for recombinant human GCD produced in transgenic plant cells in suspension, such as transgenic carrot cells.

Summary of the Invention

In a first aspect, the present invention relates to a host cell producing a high mannose recombinant protein of interest. This cell may be transformed or transfected with a recombinant nucleic acid molecule encoding a protein of interest or with an expression vector comprising said nucleic acid molecule. Such nucleic acid molecule comprises a first nucleic acid sequence encoding said protein of interest operably linked to a second nucleic acid sequence encoding a vacuolar targeting signal peptide. The first nucleic acid sequence may be optionally further operably linked to a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide. The host cell of the invention is characterized in that the protein of interest is produced by said cell in a highly mannosylated form.

The host cell of the invention may be a eukaryotic or prokaryotic cell.

In one embodiment, the host cell of the invention is a prokaryotic cell, preferably, a bacterial cell, most preferably, an *Agrobacterium tumefaciens* cell. These cells are used for infecting the preferred plant host cells described below.

In another specifically preferred embodiment, the host cell of the invention may be a eukaryotic cell, preferably, a plant cell, and most preferably, a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell.

In a specifically preferred embodiment, the plant root cell is a carrot cell. It should be noted that the transformed carrot cells of the invention are grown in suspension. As mentioned above and described in the Examples, these cells were transformed with the *Agrobacterium tumefaciens* cells.

In another embodiment, the recombinant nucleic acid molecule comprised within the host cell of the invention, comprises a first nucleic acid sequence encoding a lysosomal enzyme that is in operable linkage with a second nucleic acid sequence encoding a vacuolar targeting signal peptide derived from the basic tobacco chitinase A gene. This vacuolar signal peptide has the amino acid sequence as denoted by SEQ ID NO: 2. The first nucleic acid sequence may be optionally further linked in an operable linkage with a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide as denoted by SEQ ID NO: 1. In one embodiment, the recombinant nucleic acid molecule comprised within the host cell of the invention further comprises a promoter that is functional in plant cells. This

promoter should be operably linked to the recombinant molecule of the invention.

In another embodiment, this recombinant nucleic acid molecule may optionally further comprise an operably linked terminator which is preferably functional in plant cells. The recombinant nucleic acid molecule of the invention may optionally further comprise additional control, promoting and regulatory elements and/or selectable markers. It should be noted that these regulatory elements are operably linked to the recombinant molecule.

In a preferred embodiment, the high mannose protein of interest produced by the host cell of the invention may be a high mannose glycoprotein having exposed mannose terminal residues.

Such high mannose protein may be according to another preferred embodiment, a lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase

In a specifically preferred embodiment, the lysosomal enzyme may be the human glucocerebrosidase (GCD).

Still further, in a particular embodiment, this preferred host cell is transformed or transfected by a recombinant nucleic acid molecule which further comprises an ^{35}S promoter from Cauliflower Mosaic Virus, an octopine synthase terminator of *Agrobacterium tumefaciens* and TMV (Tobacco Mosaic Virus) omega translational enhancer element. According to a specifically

preferred embodiment, this recombinant nucleic acid molecule comprises the nucleic acid sequence substantially as denoted by SEQ ID NO: 13 and encodes a high mannose GCD having the amino acid sequence substantially as denoted by SEQ ID NO: 14.

It should be appreciated that the present invention further provides for an expression vector comprising a nucleic acid molecule encoding a biologically active lysosomal enzyme.

In one preferred embodiment, the expression vector of the invention comprises a nucleic acid molecule encoding a biologically active high mannose human glucocerebrosidase (GCD). Preferably, this preferred expression vector comprises a nucleic recombinant nucleic acid molecule which having the nucleic acid sequence substantially as denoted by SEQ ID NO: 13.

In a second aspect, the present invention relates to a recombinant high mannose protein produced by the host cell of the invention.

In a preferred embodiment, this high mannose protein may be a biologically active high mannose lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase. Most preferably, this lysosomal enzyme may be human glucocerebrosidase (GCD).

Still further, the invention provides for a recombinant biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

According to a preferred embodiment, the recombinant lysosomal enzyme of the invention can bind to a mannose receptor on a target cell in a target site. Preferably, this site may be within a subject suffering from a lysosomal storage disease.

It should be noted that the recombinant lysosomal enzyme has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme for said target cell. In a specific embodiment, the target cell at the target site may be a Kupffer cell in the liver of said subject.

In a specifically preferred embodiment, the recombinant lysosomal enzyme may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

Most preferably, this recombinant lysosomal enzyme is glucocerebrosidase (GCD).

In a third aspect, the invention relates to a method of producing a high mannose protein. Accordingly, the method of the invention comprises the steps of: (a) preparing a culture of recombinant host cells transformed or transfected with a recombinant nucleic acid molecules encoding a recombinant protein of interest or with an expression vector comprising said recombinant nucleic acid molecules; (b) culturing these host cell culture prepared by step (a) under conditions permitting the expression of said

protein, wherein said host cells produce said protein in a highly mannosylated form; (c) recovering said protein from the cells and harvesting said cells from the culture provided in (a); and (d) purifying said protein of step (c) by a suitable protein purification method.

According to a preferred embodiment, the host cell used by this method is the host cell of the invention.

In another preferred embodiment, the high mannose protein produced by the method of the invention may be a biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

This recombinant enzyme can bind to a mannose receptor on a target cell in a target site. More particularly, the recombinant enzyme produced by the method of the invention has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell. Accordingly, the target cell at the target site may be Kupffer cell in the liver of said subject.

In a specific embodiment, this lysosomal enzyme may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase. Most preferably, this lysosomal enzyme may be glucocerebrosidase (GCD).

In another preferred embodiment, the host cell used by the method of the invention may be a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell. Most preferably, the plant root cell is a carrot cell. It should be particularly noted that in the method of the invention, the transformed host carrot cells are grown in suspension.

In a further aspect, the present invention relates to a method for treating a subject having lysosomal storage disease using exogenous recombinant lysosomal enzyme, comprising: (a) providing a recombinant biologically active form of lysosomal enzyme purified from transformed plant root cells, and capable of efficiently targeting cells abnormally deficient in said lysosomal enzyme. This recombinant biologically active enzyme has exposed terminal mannose residues on appended oligosaccharides; and (b) administering a therapeutically effective amount of said recombinant biologically active lysosomal enzyme to said subject. In a preferred embodiment, the recombinant high mannose lysosomal enzyme used by the method of the invention may be produced by the host cell of the invention. Preferably, this host cell is a carrot cell.

In another specifically preferred embodiment, the lysosomal enzyme used by the method of the invention may be a high mannose enzyme comprising at least one oligosaccharide chain having an exposed mannose residue. This recombinant enzyme can bind to a mannose receptor on a target cell in a target site within a subject. More preferably, this recombinant lysosomal enzyme has increased affinity for these target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell.

More specifically, the lysosomal enzyme used by the method of the invention may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase. Preferably, this lysosomal enzyme is glucocerebrosidase (GCD).

According to a preferred embodiment, the method of the invention is therefore intended for the treatment of a lysosomal storage disease, particularly Gaucher's disease.

In such case the target cell at the target site may be a Kupffer cell in the liver of said subject.

The invention further provides for a pharmaceutical composition for the treatment of a lysosomal storage disease comprising as an active ingredient a recombinant biologically active high mannose lysosomal enzyme as defined by the invention. The composition of the invention may optionally further comprise pharmaceutically acceptable dilluent, carrier or excipient.

In a specific embodiment, the composition of the invention is intended for the treatment of Gaucher's disease. Such composition may preferably comprise as an effective ingredient a biologically active high mannose human glucocerebrosidase (GCD), as defined by the invention.

The invention further relates to the use of a recombinant biologically active high mannose lysosomal enzyme of the invention in the manufacture of a

medicament for the treatment or prevention of a lysosomal storage disease. More particularly, such disease may be Gaucher's disease.

Accordingly, this biologically active lysososomal enzyme is a biologically active high mannose human glucocerebrosidase (GCD), as defined by the invention.

The invention will be further described on the hand of the following figures, which are illustrative only and do not limit the scope of the invention which is defined by the appended claims.

Brief Description of the Figures

Figure 1A-1B

1A shows the resulting expression cassette comprising ^{35}S promoter from Cauliflower Mosaic Virus, TMV (Tobacco Mosaic Virus) omega translational enhancer element, ER targeting signal, the human GCD sequence (also denoted by SEQ ID NO: 7), vacuolar signal and octopine synthase terminator sequence from *Agrobacterium tumefaciens.*

1B shows a schematic map of pGreenII plasmid backbone.

Figure 2 shows Western blot analysis of hGCD transformed cell extracts using anti hGCD specific antibody. Standard Cerezyme (lane 1) was used as a positive control, untransformed callus was used as negative control (lane 2), various selected calli extracts are shown in lanes 3-8.

Figure 3A-3C shows the first step of purification of rhGCD on a strong cation exchange resin (Macro-Prep high-S support, Bio-Rad), packed in a XK

column (2.6x20cm). The column was integrated with an AKTA prime system (Amersham Pharmacia Biotech) that allows conductivity monitoring, pH and absorbency at 280nm. Elution of the rh-GCD was obtained with equilibration buffer containing 600mM NaCl. Fig 3A represents a standard run of this purification step. The fractions collected during the run were monitored by enzyme activity assay, as shown by Fig 3B, and tubes exhibiting enzymatic activity (in the elution peak) were pooled. Fig 3C shows coomassie-blue stain of elution fractions assayed for activity.

Figure 4A-C : shows the final purification step of the recombinant hGCD on a hydrophobic interaction resin (TSK gel, Toyopearl Phenyl-650C, Tosoh Corp.), packed in a XK column (2.6x20cm). The column was integrated with an AKTA prime system (Amersham Pharmacia Biotech) that allows conductivity monitoring, pH and absorbency at 280nm. The GCD elution pool from the previous column was loaded at 6ml/min followed by washing with equilibration buffer until the UV absorbance reach the baseline. The pure GCD was eluted by 10mM citric buffer containing 50% ethanol.
Fig 4A represents a standard run of this purification step.
Fig 4B shows the fractions collected during the run that were monitored by enzyme activity assay.
Fig 4C shows coomassie-blue stain of elution fractions assayed for activity.

Figure 5 shows activity of recombinant hGCD following uptake by peritoneal macrophages.

Figure 6 shows the effect of mannan on recombinant hGCD uptake.

Detailed Description of the Invention

Proteins for pharmaceutical use have been traditionally produced in mammalian or bacterial expression systems. In the past few years a promising new expression system was found in plants. Due to the relative simplicity of introducing new genes and potential for mass production of proteins and peptides, 'molecular pharming' is becoming increasingly popular as a protein expression system.

One of the major differences between mammalian and plant protein expression system is the variation of protein glycosylation sequences, caused by the differences in biosynthetic pathways. Glycosylation was shown to have a profound effect on activity, folding, stability, solubility, susceptibility to proteases, blood clearance rate and antigenic potential of proteins. Hence, any protein production in plants should take into consideration the potential ramifications of plant glycosylation.

Carbohydrate moiety is one of the most common post-translational modifications of proteins. Protein glycosylation is divided into two categories: N-linked and O-linked. The two types differ in amino acid to which the glycan moity is attached on protein – N-linked are attached to Asn residues, while O-linked are attached to Ser or Thr residues. In addition, the glycan sequences of each type bears unique distinguishing features. Of the two types, N-linked glycosylation is the more abundant, and its effect on proteins has been extensively studied. O-linked glycans, on other hand are relatively scarce, and less information is available regarding their influence on proteins. The majority of data available on protein glycosylation in plants focuses on N-linked. rather than O-linked glycans.

The present invention describes herein a plant expression system based on transgenic plant root cells grown in suspension. This expression system is particularly designed for efficient production of a high mannose protein of interest.

Thus, in a first aspect, the present invention relates to a host cell producing a high mannose recombinant protein of interest. This cell may be transformed or transfected with a recombinant nucleic acid molecule encoding a protein of interest or with an expression vector comprising said nucleic acid molecule. Such nucleic acid molecule comprises a first nucleic acid sequence encoding said protein of interest operably linked to a second nucleic acid sequence encoding a vacuolar targeting signal peptide. The first nucleic acid sequence may optionally further operably linked to a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide. The host cell of the invention is characterized in that the protein of interest is produced by said cell in a highly mannosylated form.

"Cells", "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cells but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generation due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. "Host cell" as used herein refers to cells which can be recombinantly transformed with naked DNA or expression vectors constructed using recombinant DNA techniques. As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., naked DNA or an

expression vector, into a recipient cells by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of the desired protein.

It should be appreciated that a drug resistance or other selectable marker is intended in part to facilitate the selection of the transformants. Additionally, the presence of a selectable marker, such as drug resistance marker may be of use in keeping contaminating microorganisms from multiplying in the culture medium. Such a pure culture of the transformed host cell would be obtained by culturing the cells under conditions which are required for the induced phenotype's survival.

As indicated above, the host cells of the invention may be transfected or transformed with a nucleic acid molecule. As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The terms should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

In yet another embodiment, the host cell of the invention may be transfected or transformed with an expression vector comprising said recombinant nucleic acid molecule. "Expression Vectors", as used herein, encompass vectors such as plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles, which enable the integration of DNA

fragments into the genome of the host. Expression vectors are typically self-replicating DNA or RNA constructs containing the desired gene or its fragments, and operably linked genetic control elements that are recognized in a suitable host cell and effect expression of the desired genes. These control elements are capable of effecting expression within a suitable host. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system. Such system typically includes a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of RNA expression, a sequence that encodes a suitable ribosome binding site, RNA splice junctions, sequences that terminate transcription and translation and so forth. Expression vectors usually contain an origin of replication that allows the vector to replicate independently of the host cell.

Plasmids are the most commonly used form of vector but other forms of vectors which serves an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels et al. Cloning Vectors: a Laboratory Manual (1985 and supplements), Elsevier, N.Y.; and Rodriquez, et al. (eds.) Vectors: a Survey of Molecular Cloning Vectors and their Uses, Buttersworth, Boston, Mass (1988), which are incorporated herein by reference.

In general, such vectors contain, in addition, specific genes which are capable of providing phenotypic selection in transformed cells. The use of prokaryotic and eukaryotic viral expression vectors to express the genes coding for the polypeptides of the present invention are also contemplated.

In one preferred embodiment, the host cell of the invention may be a eukaryotic or prokaryotic cell.

In a specific embodiment, the host cell of the invention is a prokaryotic cell, preferably, a bacterial cell, most preferably, an *Agrobacterium tumefaciens* cell. These cells are used for infecting the preferred plant host cells described below.

In another specifically preferred embodiment, the host cell of the invention may be an eukaryotic cell, preferably, a plant cell, and most preferably, a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed plant root cell, celery cell, ginger cell, horseradish cell and carrot cell.

In a specifically preferred embodiment, the plant root cell is a carrot cell. It should be noted that the transformed carrot cells of the invention are grown in suspension. As mentioned above and described in the Examples, these cells were transformed with the *Agrobacterium tumefaciens* cells of the invention.

The expression vectors or recombinant nucleic acid molecules used for transfecting or transforming the host cells of the invention may be further modified according to methods known to those skilled in the art to add, remove, or otherwise modify peptide signal sequences to alter signal peptide cleavage or to increase or change the targeting of the expressed lysosomal enzyme through the plant endomembrane system. For example, but not by way of limitation, the expression construct can be specifically engineered to target the lysosomal enzyme for secretion, or vacuolar localization, or retention in the endoplasmic reticulum (ER).

In one embodiment, the expression vector or recombinant nucleic acid molecule, can be engineered to incorporate a nucleotide sequence that encodes a signal targeting the lysosomal enzyme to the plant vacuole. For example, and not by way of limitation, the recombinant nucleic acid molecule comprised within the host cell of the invention, comprises a first nucleic acid sequence encoding a lysosomal enzyme that is in operable linkage with a second nucleic acid sequence encoding a vacuolar targeting signal peptide derived from the basic tobacco chitinase A gene. This vacuolar signal peptide has the amino acid sequence as denoted by SEQ ID NO: 2. The first nucleic acid sequence may be optionally further linked in an operable linkage with a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide as denoted by SEQ ID NO: 1. In one embodiment, the recombinant nucleic acid molecule comprised within the host cell of the invention further comprises a promoter that is functional in plant cells. This promoter should be operably linked to the recombinant molecule of the invention.

The term "operably linked" is used herein for indicating that a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame. Thus, a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules

(e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

In another embodiment, this recombinant nucleic acid molecule may optionally further comprise an operably linked terminator which is preferably functional in plant cells. The recombinant nucleic acid molecule of the invention may optionally further comprise additional control, promoting and regulatory elements and/or selectable markers. It should be noted that these regulatory elements are operably linked to the recombinant molecule.

Regulatory elements that may be used in the expression constructs include promoters which may be either heterologous or homologous to the plant cell. The promoter may be a plant promoter or a non-plant promoter which is capable of driving high levels transcription of a linked sequence in plant cells and plants. Non-limiting examples of plant promoters that may be used effectively in practicing the invention include cauliflower mosaic virus (CaMV) ^{35}S, rbcS, the promoter for the chlorophyll a/b binding protein, AdhI, NOS and HMG2, or modifications or derivatives thereof. The promoter may be either constitutive or inducible. For example, and not by way of limitation, an inducible promoter can be a promoter that promotes expression or increased expression of the lysosomal enzyme nucleotide sequence after mechanical gene activation (MGA) of the plant, plant tissue or plant cell.

The expression vectors used for transfecting or transforming the host cells of the invention can be additionally modified according to methods known to those skilled in the art to enhance or optimize heterologous gene expression in plants and plant cells. Such modifications include but are not limited to

mutating DNA regulatory elements to increase promoter strength or to alter the protein of interest.

In a preferred embodiment, the high mannose protein of interest produced by the host cell of the invention may be a high mannose glycoprotein having exposed mannose terminal residues.

Such high mannose protein may be according to another preferred embodiment, a lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase

The term "lysosomal enzyme", as used herein with respect to any such enzyme and product produced in a plant expression system described by the invention, refers to a recombinant peptide expressed in a transgenic plant cell from a nucleotide sequence encoding a human or animal lysosomal enzyme, a modified human or animal lysosomal enzyme, or a fragment, derivative or modification of such enzyme. Useful modified human or animal lysosomal enzymes include but are not limited to human or animal lysosomal enzymes having one or several naturally occurring or artificially introduced amino acid additions, deletions and/or substitutions.

Soluble lysosomal enzymes share initial steps of biosynthesis with secretory proteins, i.e., synthesis on the ribosome, binding of the N-terminal signal peptide to the surface of the rough endoplasmic reticulum (ER), transport into the lumen of the ER where the signal peptide is cleaved, and addition of oligosaccharides to specific asparagine residues (N-linked), followed by

further modifications of the nascent protein in the Golgi apparatus [von Figura and Hasilik, Annu. Rev. Biochem. 55:167-193 (1986)]. The N-linked oligosaccharides can be complex, diverse and heterogeneous, and may contain high-mannose residues. The proteins undergo further processing in a post-ER, pre-Golgi compartment and in the cis-Golgi to form either an N-linked mannose 6-phosphate (M-6-P) oligosaccharide-dependent or N-linked M-6-P oligosaccharide-independent recognition signal for lysosomal localized enzymes [Kornfeld & Mellman, Ann. Rev. Cell Biol., 5:483-525 (1989); Kaplan et al., Proc. Natl. Acad. Sci. USA 74:2026 (1977)]. The presence of the M-6-P recognition signal results in the binding of the enzyme to M-6-P receptors (MPR). These bound enzymes remain in the cell, are eventually packaged into lysosomes, and are thus segregated from proteins targeted for secretion or to the plasma membrane.

In a specifically preferred embodiment, the lysosomal enzyme may be the human glucocerebrosidase (GCD).

Still further, in a particular embodiment, this preferred host cell is transformed or transfected by a recombinant nucleic acid molecule which further comprises an ^{35}S promoter from Cauliflower Mosaic Virus, preferably, having the nucleic acid sequence as denoted by SEQ ID NO: 9, an octopine synthase terminator of *Agrobacterium tumefaciens*, preferably, having the nucleic acid sequence as denoted by SEQ ID NO: 12 and TMV (Tobacco Mosaic Virus) omega translational enhancer element. According to a specifically preferred embodiment, this recombinant nucleic acid molecule comprises the nucleic acid sequence substantially as denoted by SEQ ID NO: 13 and encodes a high mannose GCD having the amino acid sequence substantially as denoted by SEQ ID NO: 14.

It should be appreciated that the present invention further provides for an expression vector comprising a nucleic acid molecule encoding a biologically active high mannose lysosomal enzyme.

In one preferred embodiment of said aspect, the expression vector of the invention comprises a nucleic acid molecule encoding a biologically active high mannose human glucocerebrosidase (GCD). Preferably, this preferred expression vector comprises a recombinant nucleic acid molecule which having the nucleic acid sequence substantially as denoted by SEQ ID NO: 13. According to a specific embodiment, a preferred expression vector utilizes the pGREEN II plasmid as described by the following Example 1.

It should be further noted, that the invention provides for an expression cassette comprised within the expression vector described above.

In a second aspect, the present invention relates to a recombinant high mannose protein produced by the host cell of the invention.

In a preferred embodiment, this high mannose protein may be a biologically active high mannose lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase. Most preferably, this lysosomal enzyme may be human glucocerebrosidase (GCD).

The term "biologically active" is used herein with respect to any recombinant lysosomal enzyme produced in a plant expression system to mean that the

recombinant lysosomal enzyme is able to hydrolyze either the natural substrate, or an analogue or synthetic substrate of the corresponding human or animal lysosomal enzyme, at detectable levels.

Still further, the invention provides for a recombinant biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

According to a preferred embodiment, the recombinant lysosomal enzyme of the invention can bind to a mannose receptor on a target cell in a target site. Preferably, this site may be within a subject suffering from a lysosomal storage disease.

It should be noted that the recombinant lysosomal enzyme has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme for said target cell. In a specific embodiment, the target cell at the target site may be a Kupffer cell in the liver of said subject.

In a specifically preferred embodiment, the recombinant lysosomal enzyme may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

Most preferably, this recombinant lysosomal enzyme is glucocerebrosidase (GCD).

In a third aspect, the invention relates to a method of producing a high mannose protein. Accordingly, the method of the invention comprises the steps of: (a) preparing a culture of recombinant host cells transformed or transfected with a recombinant nucleic acid molecules encoding for a recombinant protein of interest or with an expression vector comprising said recombinant nucleic acid molecules; (b) culturing the host cell culture prepared by step (a) in suspension under conditions permitting the expression of said high mannose protein, wherein said host cells produce said protein in a highly mannosylated form; (c) harvesting said cells from the culture provided in (a) and recovering said protein from the cells; and (d) purifying said protein of step (c) by a suitable protein purification method.

A particular and non limiting example for recovering and purification of a high mannose protein of interest produced by the method of the invention may be found in the following Examples. The Examples show that a recombinant h-GCD produced by the invention was unexpectedly bound to internal membrane of the transformed carrot cells of the invention and not secreted to the medium. The soluble rh-GCD may be separated from cell debris and other insoluble component according to means known in the art such as filtration or precipitation. For Example, following a freeze-thaw cycle, the cells undergo breakage and release of intracellular soluble proteins, whereas the h-GCD remains bound to insoluble membrane debris. This soluble and insoluble membrane debris mixture was next centrifuged and the soluble fraction was removed thus simplifying the purification. The membrane bound h-GCD can then be dissolved by mechanical disruption in the presence of a mild detergent, protease inhibitors and neutralizing oxidation reagent. The soluble enzyme may be further purified using chromatography techniques, such as cation exchange and hydrophobic

interaction chromatography columns. During rh-GCD production in the bio-reactor and the purification process the h-GCD identity, yield, purity and enzyme activity can be determined by one or more biochemical assays. Including but not limited to detecting hydrolysis of the enzyme's substrate or a substrate analogue, SDS-polyacrylamide gel electrophoresis analysis and immunological analyses such as ELISA and Western blot.

According to a preferred embodiment, the host cell used by this method is the host cell of the invention.

In another preferred embodiment, the high mannose protein produced by the method of the invention may be a biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

This recombinant enzyme can bind to a mannose receptor on a target cell in a target site. More particularly, the recombinant enzyme produced by the method of the invention has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell. Accordingly, the target cell at the target site may be Kupffer cell in the liver of said subject.

In a specific embodiment, this lysosomal enzyme may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase. Most preferably, this lysosomal enzyme may be glucocerebrosidase (GCD).

In another preferred embodiment, the host cell used by the method of the invention may be a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell. Most preferably, the plant root cell is a carrot cell. It should be particularly noted that the transformed host carrot cells are grown in suspension.

In a further aspect, the present invention relates to a method for treating a subject, preferably a mammalian subject, having lysosomal storage disease using exogenous recombinant lysosomal enzyme, comprising: (a) providing a recombinant biologically active form of lysosomal enzyme purified from transformed plant root cells, and capable of efficiently targeting cells abnormally deficient in said lysosomal enzyme. This recombinant biologically active enzyme has exposed terminal mannose residues on appended oligosaccharides; and (b) administering a therapeutically effective amount of said recombinant biologically active lysosomal enzyme, or of composition comprising the same to said subject. In a preferred embodiment, the recombinant high mannose lysosomal enzyme used by the method of the invention may be produced by the host cell of the invention. Preferably, this host cell is a carrot cell.

By "mammalian subject" or "mammalian patient" is meant any mammal for which gene therapy is desired, including human, bovine, equine, canine, and feline subjects, most preferably, a human subject.

It should be noted that the high mannose lysosomal enzyme of the invention should be administered to the patient in need in an effective amount. As used

herein, "effective amount" means an amount necessary to achieve a selected result. For example, an effective amount of the composition of the invention useful for the treatment of a lysosomal storage disease. Administration may be oral, subcutaneous or parenteral, including intravenous, intramuscular, intraperitoneal and intranasal administration as well as intrathecal and infusion techniques. Nevertheless, most preferred methods are oral administration and injection.

In another specifically preferred embodiment, the lysosomal enzyme used by the method of the invention may be a high mannose enzyme comprising at least one oligosaccharide chain having an exposed mannose residue. This recombinant enzyme can bind to a mannose receptor on a target cell in a target site within a subject. More preferably, this recombinant lysosomal enzyme has increased affinity for these target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell. Therefore, each dose is dependent on the effective targeting of cells abnormally deficient in GCD and each dose of such form of GCD is substantially less than the dose of naturally occurring GCD that would otherwise be administered in a similar manner to achieve the therapeutic effect.

As noted above, the method of the invention is intended for the treatment of lysosomal storage diseases. Lysosomal storage diseases are a group of over 40 disorders which are the result of defects in genes encoding enzymes that break down glycolipid or polysaccharide waste products within the lysosomes of cells. The enzymatic products, e.g., sugars and lipids, are then recycled into new products. Each of these disorders results from an inherited autosomal or X-linked recessive trait which affects the levels of enzymes in the lysosome.

Generally, there is no biological or functional activity of the affected enzymes in the cells and tissues of affected individuals. In such diseases the deficiency in enzyme function creates a progressive systemic deposition of lipid or carbohydrate substrate in lysosomes in cells in the body, eventually causing loss of organ function and death. The genetic etiology, clinical manifestations, molecular biology and possibility of the lysosomal storage diseases are detailed in Scriver et al. [Scriver et al. eds., The Metabolic and Molecular Basis of Inherited Disease, 7th Ed., Vol. II, McGraw Hill, (1995)].

A few examples of lysosomal storage diseases (and their associated deficient enzymes) include Fabry disease (α-galactosidase), Farber disease (ceramidase), Gaucher disease (glucocerebrosidase), G_{m1} gangliosidosis (ß-galactosidase), Tay-Sachs disease (ß-hexosaminidase), Niemann-Pick disease (sphingomyelinase), Schindler disease (α.-N-acetylgalactosaminidase), Hunter syndrome (iduronate-2-sulfatase), Sly syndrome (ß-glucuronidase), Hurler and Hurler/Scheie syndromes (iduronidase), and I-Cell/San Filipo syndrome (mannose 6-phosphate transporter).

According to a preferred embodiment, the method of the invention is therefore intended for the treatment of a lysosomal storage disease, particularly, Gaucher's disease.

Gaucher disease is the most common lysosomal storage disease in humans, with the highest frequency encountered in the Ashkenazi Jewish population. About 5,000 to 10,000 people in the United States are afflicted with this disease [Grabowski, Adv. Hum. Genet. 21:377-441(1993)]. Gaucher disease results from a deficiency in glucocerebrosidase (hGCD; glucosylceramidase). This deficiency leads to an accumulation of the enzyme's substrate,

glucocerebroside, in reticuloendothelial cells of the bone marrow, spleen and liver, resulting in significant skeletal complications such as bone marrow expansion and bone deterioration, and also hypersplenism, hepatomegaly, thrombocytopenia, anemia and lung complications [Grabowski, (1993) ibid.; Lee, Prog. Clin. Biol. Res. 95:177-217 (1982)].

More specifically, the lysosomal enzyme used by the method of the invention may be selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase. Preferably, where the treated disease is Gaucher's disease, the lysosomal enzyme used by the method of the invention is glucocerebrosidase (GCD).

The mature hGCD polypeptide is composed of 497 amino acids and contains five N-glycosylation amino acid consensus sequences (Asn-X-Ser/Thr). Four of these sites are normally glycosylated. Glycosylation of the first site is essential for the production of active protein. Both high-mannose and complex oligosaccharide chains have been identified (Berg-Fussman et al., 1993, J. Biol. Chem. 268:14861-14866). hGCD from placenta contains 7% carbohydrate, 20% of which is of the high-mannose type (Grace & Grabowski, 1990, Biochem. Biophys. Res. Comm. 168:771-777). Treatment of placental hGCD with neuraminidase (yielding an asialo enzyme) results in increased clearance and uptake rates by rat liver cells with a concomitant increase in hepatic enzymatic activity (Furbish et al., 1981, Biochim. Biophys. Acta 673:425-434). This glycan-modified placental hGC is currently used as a therapeutic agent in the treatment of Gaucher's disease. Biochemical and site-directed mutagenesis studies have provided an initial map of regions and

residues important to folding, activator interaction, and active site location [Grace et al., J. Biol. Chem. 269:2283-2291 (1994)].

Where the method of the invention is intended for the treatment of Gaucher's disease, the target cell for the high mannose GCD of the invention may be a Kupffer cell in the liver of said treated subject.

The invention further provides for a pharmaceutical composition for the treatment of a lysosomal storage disease comprising as an active ingredient a recombinant biologically active high mannose lysosomal enzyme as defined by the invention. The composition of the invention may optionally further comprise pharmaceutically acceptable dilluent, carrier or excipient.

In a specific embodiment, the composition of the invention is intended for the treatment of Gaucher's disease. Such composition may preferably comprise as an effective ingredient a biologically active high mannose human glucocerebrosidase (GCD), as defined by the invention.

The invention further relates to the use of a recombinant biologically active high mannose lysosomal enzyme of the invention in the manufacture of a medicament for the treatment or prevention of a lysosomal storage disease. More particularly, such disease may be Gaucher's disease.

Accordingly, this biologically active lysososomal enzyme is a biologically active high mannose human glucocerebrosidase (GCD), as defined by the invention.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

Examples

Experimental procedures:

Plasmid vectors

* CE-T – Was constructed from plasmid CE obtained from Prof. Galili [United States Patent *5,367,110* November 22, (1994)]. Plasmid CE was digested with SalI. The SalI cohesive end was made blunt-ended using the large fragment of DNA polymerase I. Then the plasmid was digested with PstI and ligated to a DNA fragment coding for the ER targeting signal (SEQ ID NO: 3), a non relevant gene, and vacuolar targeting signal (SEQ ID NO: 4), digested with SmaI and PstI.

* pGREENII - obtained from Dr. P. Mullineaux [Roger P. Hellens et al., (2000) Plant Mol. Bio. 42:819-832]. Expression from the pGREEN II vector is controlled by the 35S promoter from Cauliflower Mosaic Virus, the TMV (Tobacco Mosaic Virus) omega translational enhancer element and the octopine synthase terminator sequence from *Agrobacterium tumefaciens*.

cDNA

hGCD – obtained from ATCC (Accession No. 65696), GC-2.2 [GCS-2kb; lambda-EZZ-gamma3 *Homo sapiens*] containing glucosidase beta acid [glucocerebrosidase]. Insert lengths (kb): 2.20; Tissue: fibroblast WI-38 cell.

Transformation of carrot cells and isolation of transformed cells.

Transformation of carrot cells was preformed using *Agrobacterium* transformation by an adaptation of a method described previously [Wurtele, E.S. and Bulka, K. Plant Sci. 61:253-262 (1989)]. Cells growing in liquid media were used throughout the process instead of calli. Incubation and growth times were adapted for transformation of cells in liquid culture. Briefly, *Agrobacteria* were transformed with the pGREEN II vector by electroporation [den Dulk-Ra, A. and Hooykaas, P.J. (1995) Methods Mol. Biol. 55:63-72] and then selected using 30 mg/ml paromomycine antibiotic. Carrot cells were transformed with *Agrobacteria* and selected using 60 mg/ml of paromomycine antibiotics in liquid media.

Screening of transformed carrot cells for isolation of calli expressing high levels of GCD

14 days following transformation, cells from culture were plated on solid media at dilution of 3% packed cell volume for the formation of calli from individual clusters of cells. When individual calli reached 1-2 cm in diameter, the cells were homogenized in SDS sample buffer and the resulting protein extracts were separated on SDS-PAGE [Laemmli U., (1970) Nature 227:680-685] and transferred to nitrocellulose membrane (hybond C nitrocellulose, 0.45 micron. Catalog No: RPN203C From Amersham Life Science). Western blot for detection of GCD was preformed using polyclonal anti hGCD antibodies (described herein below). Calli expressing significant levels of GCD were expanded and transferred to growth in liquid media for scale up, protein purification and analysis.

Preparation of polyclonal antibodies

75 micrograms recombinant GCD (Cerezyme™) were suspended in 3 ml complete Freund's adjuvant and injected to each of two rabbits. Each rabbit was given a booster injection after two weeks. The rabbits were bled about 10 days after the booster injection and again at one week intervals until the antibody titer began to drop. After removal of the clot the serum was divided into aliquotes and stored at -20°C.

Upscale culture growth in Metabogal's bioreactors

An about 1cm callus of genetically modified carrot cells containing the rh-GCD gene was plated onto Murashige and Skoog (MS) 9cm diameter agar medium plate containing 4.4gr/l MSD medium (Ducefa), 9.9mg/l thiamin HCl (Ducefa), 0.5mg folic acid (Sigma) 0.5mg/l biotin (Ducefa), 0.8g/l Casein hydrolisate (Ducifa), sugar 30g/l and hormones 2-4 D (Sigma). The callus was grown for 14 days at 25°C.

Suspension cell culture was prepared by sub-culturing the transformed callus in a MSD liquid medium. The suspension cells were cultivated in 250ml Erlenmeyer flask (working volume starts with 25ml and after 7 days increases to 50ml) at 25°C with shaking speed of 60rpm. Subsequently, cell culture volume was increased to 1L Erlenmeyer by addition of working volume up to 300ml under the same conditions. Inoculum of the small bio-reactor (10L) [see WO98/13469] containing 4L MSD medium, was obtained by addition of 400ml suspension cells derived from two 1L Erlenmeyer that were cultivated for seven days. After week of cultivation at 25°C with 1Lpm airflow, MDS medium was added up to 10L and the cultivation continued under the same conditions. After additional five days of cultivation, most of the cells were harvested and collected by passing the cell media through 80μ

net. The extra medium was squeezed out and the packed cell cake was store at –70°C.

Protein purification

In order to separate the medium from the insoluble GCD, frozen cell cake containing about 100g wet weight cells was thawed, followed by centrifugation of the thawed cells at 17000xg for 20min at 4°C. The insoluble materials and intact cells were washed by re-suspension in 100ml washing buffer (20mM sodium phosphate pH 7.2, 20mM EDTA), and then precipitated by centrifugation at 17000g for 20min at 4°C. The rh-GCD (recombinant human GCD) was extracted and solubilized by homogenization of the pellet in 200ml extraction buffer (20mM sodium phosphate pH 7.2, 20mM EDTA, 1mM PMSF, 20mM ascorbic acid, 3.8g polyvinylpolypyrrolidone (PVPP), 1mM DTT and 1% Triton-x-100). The homogenate was then shaken for 30min at room temperature and clarified by centrifugation at 17000xg for 20min at 4°C. The pellet was discarded and the pH of the supernatant was adjusted to pH 5.5 by addition of concentrated citric acid. Turbidity generated after pH adjustment was clarified by centrifugation under the same conditions described above.

Further purification was performed by chromatography columns procedure as follows: 200ml of clarified medium were loaded on 20ml strong cation exchange resin (Macro-Prep high-S support, Bio-Rad) equilibrated in 25mM sodium citrate buffer pH 5.5, packed in a XK column (2.6x20cm). The column was integrated with an AKTA (prime system (Amersham Pharmacia Biotech) that allowed to monitor the conductivity, pH and absorbency at 280nm. The sample was loaded at 20ml/min, afterwards the column was washed with equilibration buffer (25mM sodium citrate buffer pH 5.5) at flow rate of

12ml/min until UV absorbency reached the base line. Pre-elution of the rh-GCD was performed with equilibration buffer containing 200mM NaCl and the elution was obtained with equilibration buffer containing 600mM NaCl. Fractions collected during the run were monitored by enzyme activity assay, and tubes exhibiting enzymatic activity (in the elution peak) were pooled. Pooled samples were diluted (1:5) in water containing 5% ethanol and pH adjusted to 6.0 with NaOH. Sample containing the rh-GCD was applied on the second XK column (1.6x20cm) packed with 10ml of the same resin as in the previous column. The resin in this column was equilibrate with 20mM citrate buffer pH 6.0 containing 5% ethanol. Following the sample load the column was washed with the equilibration buffer and the GCD was eluted from the column by elution buffer (20mM citrate buffer pH 6.0, 5% ethanol and 1M NaCl). The fractions of the absorbent peak in the elution step were pooled and applied on a third column.

The final purification step was performed on a XK column (1.6x20cm) packed with 8ml hydrophobic interaction resin (TSK gel, Toyopearl Phenyl-650C, Tosoh Corp.). The resin was equilibrated in 10mM citrate buffer pH 6.0 containing 5% ethanol. The GCD elution pool from the previous column was loaded at 6ml/min followed by washing with equilibration buffer until the UV absorbent reach the baseline. The pure GCD was eluted by 10mM citric buffer containing 50% ethanol, pooled and stored at -20^{0}C.

Determination of protein concentration

Protein concentrations in cell extracts and fractions were assayed by the method of Lowry/Bradford (Bio Rad protein assay) [Bradford, M., Anal. Biochem. (1976) 72:248] using a bovine serum albumin standard (fraction V Sigma). Alternatively, concentration of homogenous protein samples was

determined by absorption at 280 nm, 1mg/ml=1.4 $O.D_{280}$. Purity was determined by 280/260nm ratio.

GCD enzyme activity assay

Enzymatic activity of GCD was determined using p-nitrophenyl-β-D-glucopyranoside (Sigma) as a substrate. Assay buffer contained 60mM phosphate-citrate buffer pH=6, 4mM b-mercaptoethanol, 1.3mM EDTA, 0.15% Triton X-100, 0.125% sodium taurocholate. Assay was preformed in 96 well ELISA plates, 0-50 microliter of sample were incubated with 250 microliter assay buffer and substrate was added to final concentration of 4mM. The reaction was incubated at 37°C for 60min. Product (p-nitrophenyl; pNP) formation was detected by absorbance at 405nm. Absorbance at 405nm was monitored at t=0 and at the end point. After 60 min, 6 microliter of 5N NaOH were added to each well and absorbance at 405 nm was monitored again. Reference standard curve assayed in parallel, was used to quantitate concentrations of GCD in the tested samples [Friedman *et al.*, (1999) Blood, 93(9):2807-16].

Biochemical analyses:

In gel proteolysis and mass spectrometry analysis

The stained protein bands in the gel were cut with a clean razor blade and the proteins in the gel were reduced with 10mM DTT and modified with 100 mM iodoacetamide in 10mM ammonium bicarbonate. The gel pieces were treated with 50% acetonitrile in 10 mM ammonium bicarbonate to remove the stain from the proteins following by drying the gel pieces. The dried gel pieces were rehydrated with 10% acetonitrile in 10 mM ammonium bicarbonate containing about 0.1 μg trypsin per sample. The gel pieces were

incubated overnight at 37°C and the resulting peptides were recovered with 60% acetonitrile with 0.1% trifluoroacetate.

The tryptic peptides were resolved by reverse-phase chromatography on 0.1 X 300-mm fused silica capillaries (J&W, 100 micrometer ID) home-filled with porous R2 (Persepective). The peptides were eluted using a 80-min linear gradient of 5 to 95% acetonitrile with 0.1% acetic acid in water at flow rate of about 1 µl/min. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnegan, San Jose, CA). Mass spectrometry was performed in the positive ion mode using repetitively full MS scan followed by collision induces dissociation (CID) of the most dominant ion selected from the first MS scan. The mass spectrometry data was compared to simulated proteolysis and CID of the proteins in the NR-NCBI database using the Sequest software [J. Eng and J. Yates, University of Washington and Finnegan, San Jose].

The amino terminal of the protein was sequenced on Peptide Sequencer 494A (Perkin Elmer) according to manufacture instructions.

GCD Uptake of peritoneal macrophages

Targeting and uptake of GCD to macrophages is known to be mediated by the Mannose/N-acetylglucosmine receptor and can be determined using thioglycolate-elicited peritoneal macrophages obtained from mice, as described by Stahl P. *and Gordon S.* [J. Cell Biol. (1982) 93(1):49-56]. Briefly, mice (female, strain C57-B6) were injected intraperitoneally with 2.5 ml of 2.4% Bacto-thioglycolate medium w/o dextrose (Difco Cat. No. 0363-17-2). After 4-5 days, treated mice were sacrificed by cervical dislocation and the peritoneal cavity rinsed with phosphate buffered saline. Cells were pelleted by centrifugation (1000xg 10 min) and were resuspended in DMEM (Beit

Haemek, Israel) containing 10% fetal calf serum. Cells were then plated at 1-2x10^5 cell/well in 96-well tissue culture plates and incubated at 37°C. After 90 minutes, non-adherent cells were washed out three times using PBS, and the adherent macrophages were incubated for 90 min at 37°C, in culture medium containing specified quantities of rhGCD, ranging from 0 to 40 micrograms in 200 microliter final volume, in the absence and presence of yeast mannan (2-10 ,5 mg/ml). After incubation, medium containing excess rhGCD was removed, and cells were washed three times with PBS and then lysed with RIPA buffer. The amount of rhGCD taken up by the cells was determined by subjecting the cell lysates to *in vitro* glycosidase assay as described above.

Example 1

Construction of expression plasmid

The cDNA coding for hGCD (ATTC clone number 65696) was amplified using the forward: 5' CAGAATTCGCCCGCCCCTGCA 3' (also denoted by SEQ ID NO: 1) and the reverse: 5' CTCAGATCTTGGCGATGCCACA 3' (also denoted by SEQ ID NO: 2) primers.

The purified PCR DNA product was digested with endonucleases EcoRI and BglII (see recognition sequences underlined in the primers) and ligated into an intermediate vector having an expression cassette CE-T digested with the same enzymes. CE-T includes ER targeting signal MKTNLFLFLIFSLLLSLSSAEA (also denoted by SEQ ID NO: 3) from the basic endochitinase gene [*Arabidopsis thaliana*], and vacuolar targeting signal from Tobacco chitinase A: DLLVDTM* (also denoted by SEQ ID NO: 4).

The expression cassette was cut and eluted from the intermediate vector and ligated into the binary vector pGREENII using restriction enzymes SmaI and XbaI, forming the final expression vector. Kanamycine resistance is conferred by the NPTII gene driven by the nos promoter obtained together with the pGREEN vector (Fig. 1B). The resulting expression cassette is presented by Fig. 1A.

The resulting plasmid was sequenced to ensure correct in-frame fusion of the signals using the following sequencing primers:
Primer from the 5' 35S promoter: 5' CTCAGAAGACCAGAGGGC 3' (also denoted by SEQ ID NO: 5), and the 3' terminator: 5' CAAAGCGGCCATCGTGC 3' (also denoted by SEQ ID NO: 6). The verified cloned hGCD coding sequence is denoted by SEQ ID NO: 7.

Example 2

Transformation of carrot cells and screening for transformed cells expressing rhGCD.

Transformation of carrot cells was performed by Agrobacterium transformation as described previously by [Wurtele and Bulka (1989) *ibid.*]. Genetically modified carrot cells were plated onto Murashige and Skoog (MS) agar medium with antibiotics for selection of transformants. As shown by Fig. 2, extracts prepared from arising calli were tested for expression of GCD by Western blot analysis using anti hGCD antibody, and were compared to Cerezyme standard (positive control) and extracts of non-transformed cells (negative control). Of the various calli tested, one callus (number 22) was selected for scale-up growth and protein purification.

Upscale culture growth in bioreactors

Suspension cultures of callus 22 were obtained by sub-culturing of transformed callus in a liquid medium. Cells were cultivated in shaking Erlenmeyer flasks, until total volume was sufficient for inoculating the bioreactor (as described in Experimental procedures). The genetically modified transgenic carrot cells can be cultivated over months, and cell harvest can be obtained in cycling of 5 to 7 days (data not shown). At the seventh cultivation day, when the amount of rh-GCD production in carrot cell is at the peak, cells were harvested by passing of culture through 100mesh nets. It should be noted that cells may be harvested by means known in the art such as filtration or centrifugation. The packed cell cake, which provides the material for purification of h-GCD to homogeneity, can be stored at freezing temperature.

Example 3

Purification of recombinant active hGCD protein from transformed carrot cells

Recombinant h-GCD expressed in transformed carrot cells was found to be bound to internal membranes of the cells and not secreted to the medium. Mechanically cell disruption leaves the rGCD bound to insoluble membrane debris (data not shown). rGCD was then dissolved using mild detergents, and separated from cell debris and other insoluble components. The soluble enzyme was further purified using chromatography techniques, including cation exchange and hydrophobic interaction chromatography columns as described in Experimental procedures.

Fig. 3 represents the first step of purification on a strong cation exchange resin (Macro-Prep high-S support, Bio-Rad). Elution of the rh-GCD was obtained with equilibration buffer containing 600mM NaCl. Fig. 3A represents a standard run of this purification step. The fractions collected during the run were monitored by enzyme activity assay, as shown by Fig. 3B, and Fig. 3C shows coomassie-blue stain of elution fractions assayed for activity.

In a batch purification of 0.5kg (w/w cells) that were processed containing about 500μg GCD at the starting point, about 180μg were purified to the level greater than 80%.

Fig. 4 represents the final step of purification on a hydrophobic interaction resin (TSK gel, Toyopearl Phenyl-650C, Tosoh Corp.). The GCD elution pool from the previous column was loaded and the pure GCD was eluted by 10mM citric buffer containing 50% ethanol. Fig. 4A represents a standard run of this purification step. The fractions collected during the run were monitored by enzyme activity assay (Fig. 4B), and Fig. 4C shows coomassie-blue stain of elution fractions assayed for activity.

Biochemical analysis

To validate the identity of purified rhGCD, Mass-Spec Mass-Spec (MSMS) analysis was preformed. Results obtained showed 49% coverage of protein sequence that matched the predicted amino acid sequence, based on the DNA of the expression cassette, including the leader peptide and targeting sequences.

Uptake and activity of recombinant hGCD in peritoneal macrophages

To determine whether the rhGCD produced in carrot has been correctly glycosylated and can undergo uptake by target cells, and thus be useful for treatment of Gaucher's disease, the ability of the rhGCD to bind to and be taken up by macrophages was next assayed. Targeting of rhGCD to macrophages is mediated by the Mannose/N-acetylglucosamine (Man/GlcNAc) receptor and can be determined using thioglycolate-elicited peritoneal macrophages. As shown by Fig. 5, rGCD undergoes uptake by cells at comparable levels with Cerezyme.

Furthermore, as shown by Fig. 6, addition of mannan clearly inhibited binding of rGCD by the cells. At concentration of 2mg/ml of mannan, the binding of rGCD was inhibited by 50%.

These results show that even without remodeling of glycan structures, rhGCD expressed and purified from transformed carrot cells can undergo uptake to target macrophage cells specifically through Man/GlcNAc receptors. Moreover, this recombinant rhGCD is enzymatically active.

SEQUENCE LISTING

SEQ ID NO: 1
Amino acid sequence of the Signal Peptide ER:

MKTNLFLFLIFSLLLSLSSAEF

SEQ ID NO: 2
Amino acid sequence of the Vacuolar targeting signal from Tobacco chitinase A:

DLLVDTM

SEQ ID NO: 3
Nucleic acid sequence of the Forward primer:

cagaattcgcccgcccctgca

SEQ ID NO: 4
Nucleic acid sequence of theReverse primer:

ctcagatcttggcgatgccaca

SEQ ID NO: 5
Nucleic acid sequence of the forward primer from the 35S promoter:

ctcagaagaccagagggct

SEQ ID NO: 6
Nucleic acid sequence of the backward primer from the terminator:

caaagcggccatcgtgc

SEQ ID NO: 7
Nucleic acid sequence of the human GCD cDNA used for the constructs of the invention

gcccgccc ctgcatccct aaaagcttcg gctacagctc ggtggtgtgt
gtctgcaatg ccacatactg tgactccttt gacccccga cctttcctgc ccttggtacc ttcagccgct atgagagtac acgcagtggg cgacggatgg agctgagtat ggggcccatc caggctaatc acacgggcac aggcctgcta ctgaccctgc agccagaaca gaagttccag aaagtgaagg gatttggagg ggccatgaca gatgctgctg ctctcaacat ccttgccctg tcacccccctg cccaaaattt gctacttaaa tcgtacttct ctgaagaagg aatcggatat aacatcatcc gggtacccat ggccagctgt gacttctcca tccgcaccta cacctatgca gacacccctg atgatttcca gttgcacaac ttcagcctcc cagaggaaga taccaagctc aagatacccc tgattcaccg agccctgcag ttggcccagc gtcccgtttc actccttgcc agcccctgga catcacccac ttggctcaag accaatggag cggtgaatgg gaaggggtca ctcaagggac agcccggaga catctaccac cagacctggg ccagatactt tgtgaagttc ctggatgcct atgctgagca caagttacag ttctgggcag tgacagctga aaatgagcct tctgctgggc tgttgagtgg atacccctc cagtgcctgg gcttcacccc tgaacatcag cgagacttca ttgcccgtga cctaggtcct accctcgcca acagtactca ccacaatgtc cgcctactca tgctggatga ccaacgcttg ctgctgcccc actgggcaaa ggtggtactg acagacccag aagcagctaa atatgttcat ggcattgctg tacattggta cctggacttt ctggctccag ccaaagccac cctaggggag acacaccgcc tgttccccaa caccatgctc tttgcctcag aggcctgtgt gggctccaag ttctgggagc agagtgtgcg gctaggctcc tgggatcgag ggatgcagta cagccacagc atcatcacga acctcctgta ccatgtggtc ggctggaccg actggaacct tgccctgaac cccgaaggag gacccaattg ggtgcgtaac tttgtcgaca gtcccatcat tgtagacatc accaaggaca cgttttacaa acagcccatg ttctaccacc ttggccactt cagcaagttc attcctgagg gctcccagag agtggggctg gttgccagtc agaagaacga cctggacgca gtggcactga tgcatcccga tggctctgct gttgtggtcg tgctaaaccg ctcctctaag gatgtgcctc ttaccatcaa ggatcctgct gtgggcttcc tggagacaat ctcacctggc tactccattc acacctacct gtggcatcgc cag

SEQ ID NO: 8
Glucocerebrosidase amino acid sequence

A R P C I P K S F G Y S S V V
C V C N A T Y C D S F D P P T F P A L G T F S
R Y E S T R S G R R M E L S M G P I Q A N H T
G T G L L L T L Q P E Q K F Q K V K G F G G A
M T D A A A L N I L A L S P P A Q N L L L K S
Y F S E E G V R L L M L N D Q R L L L P H W A K V V L T
D P E A A K Y V H G I A V H W Y L D F L A P A K A T L G
E T H R L F P N T M L F A S E A C V G S K F W E Q S V R
L G S W D R G M Q Y S H S I I T N L L Y H V V G W T D W
N L A L N P E G G P N W V R N F V D S P I I V D I T K D T
F Y K Q P M F Y H L G H F S K F I P E G S Q R V G L V A
S Q K N D L D A V A L M H P D G S A V V V V L N R S S K
D V P L T I K D P A V G F L E T I S P G Y S I H T Y L W H
R Q

SEQ ID NO: 9
^{35}S Promoter nucleic acid sequence

Ttttcacaaagggtaatatcgggaaacctcctcggattccattgcccagctatctgtcacttcatcgaaaggac
agtagaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggctatcgttcaagatgcctc
taccgacagtggtcccaaagatggacccccacccacgaggaacatcgtggaaaaagaagacgttccaaccac
gtcttcaaagcaagtggattgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgc
aagacccttcctctatataaggaagttcatttcatttggagaggac

SEQ ID NO: 10
Nucleic acid sequence encoding the ER signal peptide

atgaagactaatctttttctctttctcatctttcacttctc ctatcattatcctcggccgaattc

SEQ ID NO: 11
Nucleic acid sequence encoding the vacuolar targeting sequence

gatcttttagtcgatactatg

SEQ ID NO: 12
Nucleic acid sequence of the terminator

taatttcatgatctgttttgttgtattcccttgcaatgcagggcctagggctatgaAtaaagttaatgtgtgaat
gtgtgaatgtgtgattgtgacctgaagggatcacgactataatcgtttataataaacaaagactttgtcccaaa
aaccccccccccngcaga

SEQ ID NO: 13
Nucleic acid sequence of the expression cassette of the invention

ttttcacaaagggtaatatcgggaaacctcctcggattccattgcccagctatctgtcacttcatcgaaaggaca
gtagaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggctatcgttcaagatgcctct
accgacagtggtcccaaagatggacccccacccacgaggaacatcgtggaaaaagaagacgttccaaccacg
tcttcaaagcaagtggattgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgca
agacccttcctctatataaggaagttcatttcatttggagaggacaggcttcttgagatccttcaacaattacca
acaacaacaaacaacaaacaacattacaattactatttacaattacagtcgagggatccaaggagatataac

aatgaagactaatctttttctctttctcatctttcacttctcctatcattatcctcggccgaattcgcccgcccctgc
atccctaaaagcttcggctacagctcggtggtgtgtgtctgcaatgccacatactgtgactcctttgacccccga
cctttcctgcccttggtaccttcagccgctatgagagtacacgcagtgggcgacggatggagctgagtatgggg
cccatccaggctaatcacacgggcacaggcctgctactgaccctgcagccagaacagaagttccagaaagtga
agggatttggaggggccatgacagatgctgctgctctcaacatccttgccctgtcacccccctgcccaaaatttgc
tacttaaatcgTacttctctgaagaaggaatcggatataacatcatccgggtacccatggccagctgtgacttc
tccatccgcacctacacctatgcagacacccctgatgatttccagttgcacaacttcagcctcccagaggaagat
accaagctcaagataccctgattcaccgagccctgcagttggcccagcgtcccgtttcactccttgccagcccct
ggacatcacccacttggctcaagaccaatggagcggtgaatgggaaggggtcactcaagggacagcccgga
gacatctaccaccagacctgggccagatactttgtgaagttcctggatgcctatgctgagcacaagttacagttc
tgggcagtgacagctgaaaatgagccttctgctgggctgttgagtggataccccttccagtgcctgggcttcacc
cctgaacatcagcgagacttcattgcccgtgacctaggtcctaccctcgccaacagtactcaccacaatgtccgc
ctactcatgctggatgaccaacgcttgctgctgccccactgggcaaaggtggtactgacagacccagaagcag
ctaaatatgttcatggcattgctgtacattggtacctggactttctggctccagccaaagccaccctaggggaga
cacaccgcctgttccccaacaccatgctctttgcctcagaggcctgtgtgggctccaagttctgggagcagagtg
tgcggctaggctcctgggatcgagggatgcagtacagccacagcatcatcacgaacctcctgtaccatgtggtc
ggctggaccgactggaaccttgccctgaaccccgaaggaggacccaattgggtgcgtaactttgtcgacagtcc
catcattgtagacatcaccaaggacacgtttacaaacagcccatgttctaccaccttggccacttcagcaagtt
cattcctgagggctcccagagagtggggctggttgccagtcagaagaacgacctggacgcagtggcactgatg
catcccgatggctctgctgttgtggtcgtgctaaaccgctcctctaaggatgtgcctcttaccatcaaggatcctg
ctgtgggcttcctggagacaatctcacctggctactccattcacacctacctgtggcatcgccaagatcttttagt
cgatactatgtaatttcatgatctgttttgttgtattcccttgcaatgcagggcctagggctatgaAtaaagtta
atgtgtgaatgtgtgaatgtgtgattgtgacctgaagggatcacgactataatcgtttataataaacaaagac
tttgtcccaaaaaccccccccccngcaga

SEQ ID NO: 14
Amino acid sequence of the recombinant protein of the invention

M K T N L F L F L I F S L L L S L S S A E F A R P C I P K
S F G Y S S V V C V C N A T Y C D S F D P P T F P A L G T
F S R Y E S T R S G R R M E L S M G P I Q A N H T G T G
L L L T L Q P E Q K F Q K V K G F G G A M T D A A A L N
I L A L S P P A Q N L L L K S Y F S E E G I G Y N I I R V P
M A S C D F S I R T Y T Y A D T P D D F Q L H N F S L P
E E D T K L K I P L I H R A L Q L A Q R P V S L L A S P
W T S P T W L K T N G A V N G K G S L K G Q P G D I Y H
Q T W A R Y F V K F L D A Y A E H K L Q F W A V T A E N
E P S A G L L S G Y P F Q C L G F T P E H Q R D F I A R D
L G P T L A N S T H H N V R L L M L D D Q R L L L P H W
A K V V L T D P E A A K Y V H G I A V H W Y L D F L A P

A K A T L G E T H R L F P N T M L F A S E A C V G S K F
W E Q S V R L G S W D R G M Q Y S H S I I T N L L Y H V
V G W T D W N L A L N P E G G P N W V R N F V D S P I I
V D I T K D T F Y K Q P M F Y H L G H F S K F I P E G S
Q R V G L V A S Q K N D L D A V A L M H P D G S A V V V
V L N R S S K D V P L T I K D P A V G F L E T I S P G Y S
I H T Y L W H R Q D L L V D T M

Claims

1. A host cell producing a high mannose recombinant protein of interest, which cell is transformed or transfected with a recombinant nucleic acid molecule encoding a protein of interest or with an expression vector comprising said nucleic acid molecule, wherein said nucleic acid molecule comprises a first nucleic acid sequence encoding said protein of interest operably linked to a second nucleic acid sequence encoding a vacuolar targeting signal peptide, said first nucleic acid sequence being optionally further operably linked to a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide, characterized in that the protein of interest is produced by said cell in a highly mannosylated form.

2. The host cell according to claim 1, wherein said host cell is any one of a eukaryotic and a prokaryotic cell.

3. The host cell according to claim 2, wherein said prokaryotic cell is a bacterial cell, preferably an *Agrobacterium tumefaciens* cell.

4. The host cell according to claim 2, wherein said eukaryotic cell is a plant cell.

5. The host cell according to claim 4, wherein said plant cell is a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell.

6. The host cell according to claim 5, wherein said plant root cell is a carrot cell.

7. The host cell according to claim 6, wherein said recombinant nucleic acid molecule comprises a first nucleic acid sequence encoding said protein of interest that is in operable link with a second nucleic acid sequence encoding a vacuolar targeting signal peptide derived from the basic tobacco chitinase A gene, which vacuolar signal peptide has the amino acid sequence as denoted by SEQ ID NO: 2, wherein said first nucleic acid sequence is optionally further operably linked to a third nucleic acid sequence encoding an ER (endoplasmic reticulum) targeting signal peptide as denoted by SEQ ID NO: 1.

8. The host cell according to claim 7, wherein said recombinant nucleic acid molecule further comprises a promoter that is functional in plant cells, wherein said promoter is operably linked to said recombinant molecule.

9. The host cell according to claim 8, wherein said recombinant nucleic acid molecule further comprises a terminator that is functional in plant cells, wherein said terminator is operably linked to said recombinant molecule.

10. The host cell according to claim 9, wherein said recombinant nucleic acid molecule optionally further comprises additional control, promoting and regulatory elements and/or selectable markers, wherein said regulatory elements are operably linked to said recombinant molecule.

11. The host cell according to claim 10, wherein said high mannose protein is a high mannose glycoprotein having exposed mannose terminal residues.

12. The host cell according to claim 11, wherein said high mannose protein is a biologically active high mannose lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase

13. The host cell according to claim 12, wherein said lysosomal enzyme is human glucocerebrosidase (GCD).

14. The host cell according to claim 13, wherein said GCD comprises the amino acid sequence substantially as denoted by SEQ ID NO: 8, encoded by the nucleic acid sequence as denoted by SEQ ID NO: 7.

15. The host cell according to claim 14, wherein said cell is transformed or transfected with a recombinant nucleic acid molecule or with an expression vector comprising said molecule, which recombinant nucleic acid molecule further comprises an ^{35}S promoter from Cauliflower Mosaic Virus, an octopine synthase terminator of *Agrobacterium tumefaciens*, and the regulatory element is the TMV (Tobacco Mosaic Virus) omega translational enhancer element, and having the nucleic acid sequence substantially as denoted by SEQ ID NO: 13 encoding

GCD having the amino acid sequence substantially as denoted by SEQ ID NO: 14. .

16. A recombinant high mannose protein produced by the host cell according to any one of claims 1 to 15.

17. The recombinant high mannose protein according to claim 16, wherein said high mannose protein is a biologically active high mannose lysosomal enzyme selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase.

18. The recombinant protein according to claim 17, wherein said lysosomal enzyme is human glucocerebrosidase (GCD).

19. A recombinant biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

20. The recombinant lysosomal enzyme according to claim 19, wherein said recombinant enzyme can bind to a mannose receptor on a target cell in a target site within a subject suffering from a lysosomal storage disease.

21. The recombinant lysosomal enzyme according to claim 20, wherein said recombinant lysosomal enzyme has increased affinity for said

target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme for said target cell.

22. The recombinant lysosomal enzyme according to claim 21, wherein said recombinant lysosomal enzyme is selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

23. The recombinant lysosomal enzyme according to claim 22, wherein said recombinant lysosomal enzyme is glucocerebrosidase (GCD).

24. The recombinant lysosomal enzyme according to claim 23, wherein said target cell at the target site is a Kupffer cell in the liver of said subject.

25. A method of producing a high mannose protein comprising the steps of:

(a) preparing a culture of recombinant host cells transformed or transfected with a recombinant nucleic acid molecules encoding for a recombinant protein of interest or with an expression vector comprising said recombinant nucleic acid molecules;

(b) culturing said host cell culture prepared by step (a) in suspension under conditions permitting the expression of said protein, wherein said host cells produce said protein in a highly mannosylated form;

(c) harvesting said cells from the culture provided in (a) and recovering said protein from the cells; and

(d) purifying said protein of step (c) by a suitable protein purification method.

26. The method according to claim 25, wherein said host cell is as defined by any one of claims 1 to 15.

27. The method according to any one of claims 25 and 26, wherein said high mannose protein is a biologically active high mannose lysosomal enzyme having at least one oligosaccharide chain comprising an exposed mannose residue.

28. The method according to claim 27, wherein said recombinant enzyme binds to a mannose receptor on a target cell in a target site.

29. The method according to claim 28, wherein said recombinant enzyme has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell.

30. The method according to claim 29, wherein said lysosomal enzyme is selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase and sialidase.

31. The method molecule according to claim 30, wherein said lysosomal enzyme is glucocerebrosidase (GCD).

32. The method according to claim 31, wherein said target cell at the target site is Kupffer cell in the liver of said subject.

33. The method according to claim 32, wherein said host cell is a plant root cell selected from the group consisting of *Agrobacterium rihzogenes* transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell.

34. The method according to claim 33, wherein said plant root cell is a carrot cell.

35. The method according to claim 34, wherein said transformed host carrot cells are grown in suspension.

36. A method for treating a subject having lysosomal storage disease using exogenous recombinant lysosomal enzyme, comprising:

(a) providing a recombinant biologically active form of lysosomal enzyme purified from transformed plant root cells, and capable of efficiently targeting cells abnormally deficient in said lysosomal enzyme, wherein the recombinant biologically active enzyme has exposed terminal mannose residues on appended oligosaccharides; and

(b) administering a therapeutically effective amount of said recombinant biologically active lysosomal enzyme to said subject.

37. The method according to claim 36, wherein said host cell is as defined by claims 1 to 15.

38. The method according to claim 36, wherein said host cell is a carrot cell.

39. The method according to claim 38, wherein said lysosomal enzyme is a high mannose enzyme comprising at least one oligosaccharide chain having an exposed mannose residue.

40. The method according to claim 39, wherein said recombinant enzyme can bind to a mannose receptor on a target cell in a target site within a subject.

41. The method according to claim 40, wherein said recombinant lysosomal enzyme has increased affinity for said target cell, in comparison with the corresponding affinity of a naturally occurring lysosomal enzyme to said target cell.

42. The method according to claim 41, wherein said lysosomal enzyme is selected from the group consisting of glucocerebrosidase (GCD), acid sphingomyelinase, hexosaminidase, α-N-acetylgalactosaminidise, acid lipase, α-galactosidase, glucocerebrosidase, α-L-iduronidase, iduronate sulfatase, α-mannosidase or sialidase.

43. The method according to claim 42, wherein said lysosomal enzyme is glucocerebrosidase (GCD).

44. The method according to claim 43, wherein said lysosomal storage disease is Gaucher's disease.

45. The method according to claim 44, wherein said target cell at the target site is a Kupffer cell in the liver of said subject.

46. A pharmaceutical composition for the treatment of a lysosomal storage disease comprising as an active ingredient a recombinant biologically active high mannose lysosomal enzyme as defined by any one of claims 16 to 24, which composition optionally further comprises pharmaceutically acceptable dilluent, carrier or excipient.

47. The composition according to claim 46, wherein said lysosomal storage disease is Gaucher's disease.

48. The composition according to claim 47, wherein said recombinant lysosomal enzyme is a biologically active high mannose human glucocerebrosidase (GCD), as defined by claim 23.

49. Use of a recombinant biologically active high mannose lysosomal enzyme as defined by any one of claims 17 to 24, in the manufacture of a medicament for the treatment or prevention of a lysosomal storage disease.

50. The use according to claim 41, wherein said disease is Gaucher's disease.

51. The use according to claim 50, wherein said biologically active lysososomal enzyme is a biologically active high mannose human glucocerebrosidase (GCD), as defined by claim 23 .

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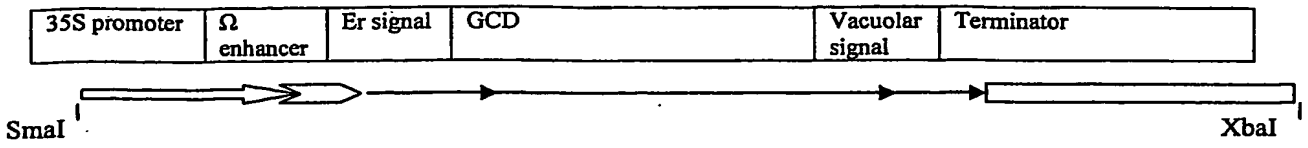

Fig. 1A

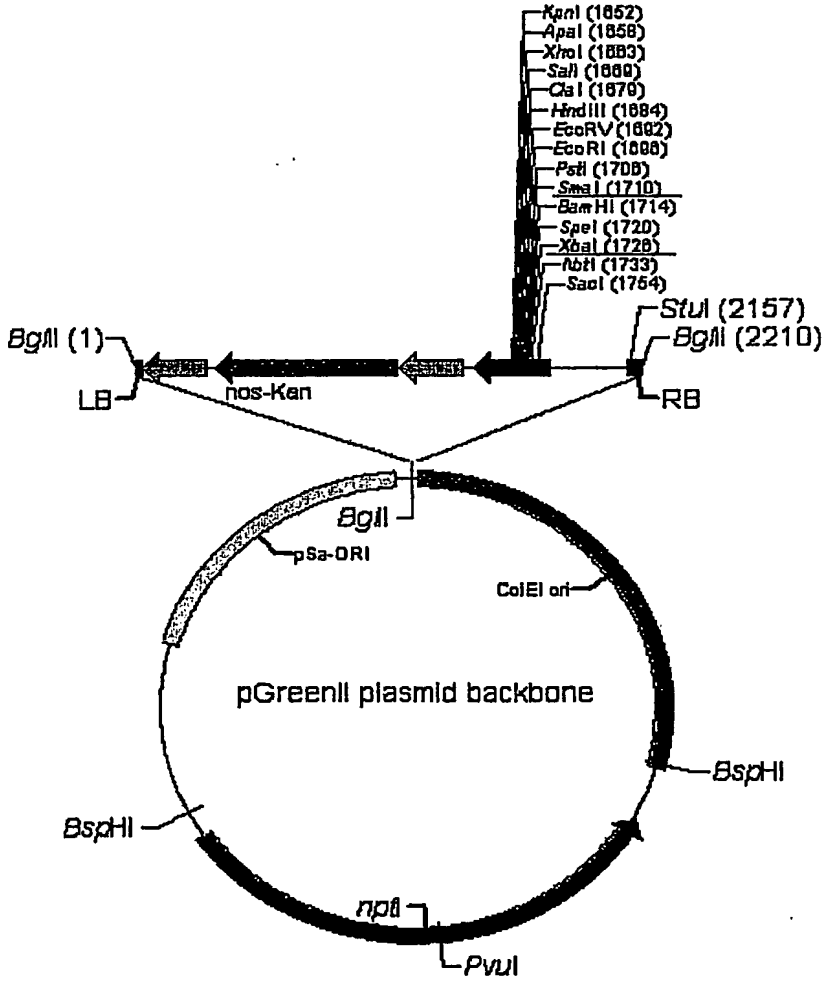

Fig. 1B

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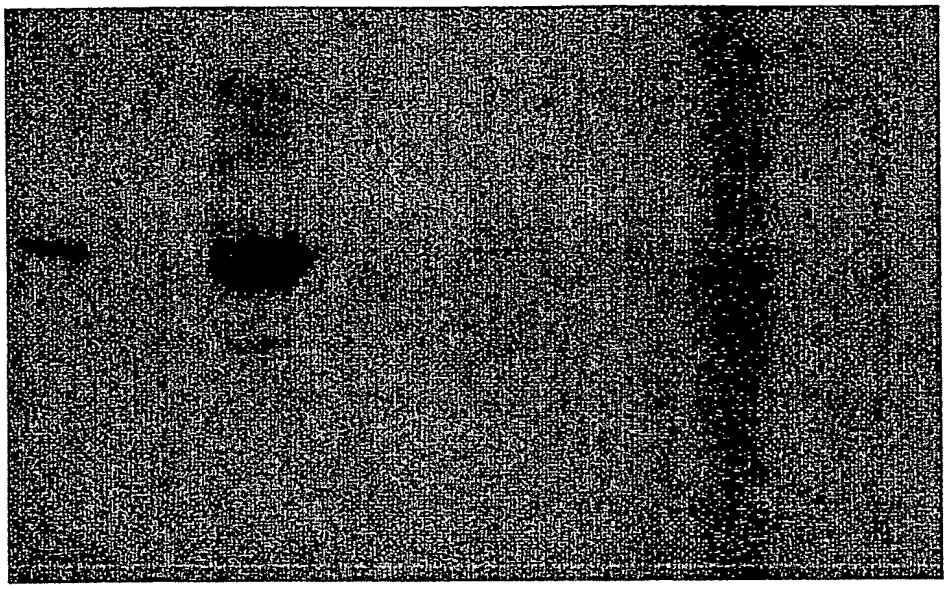

Fig. 2

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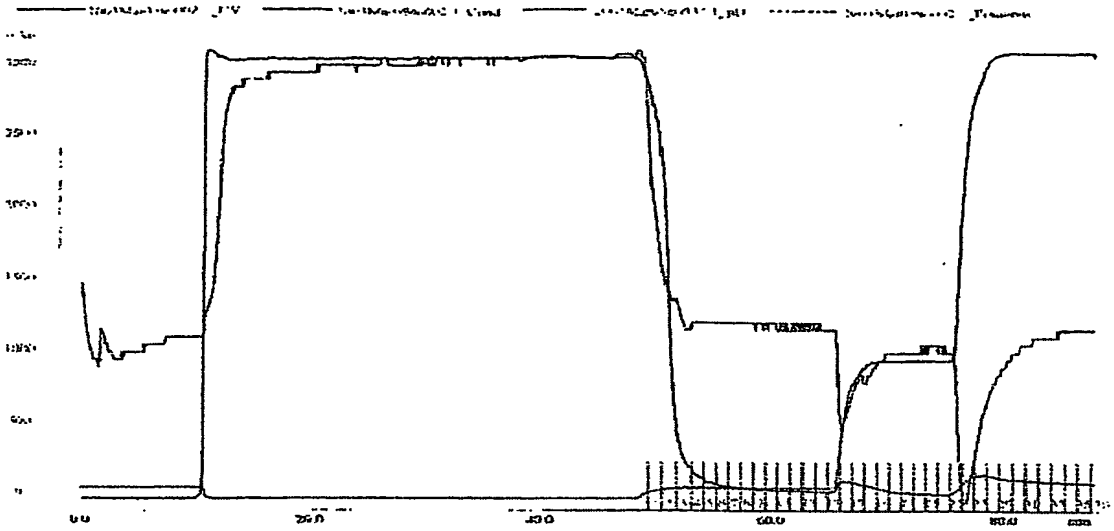

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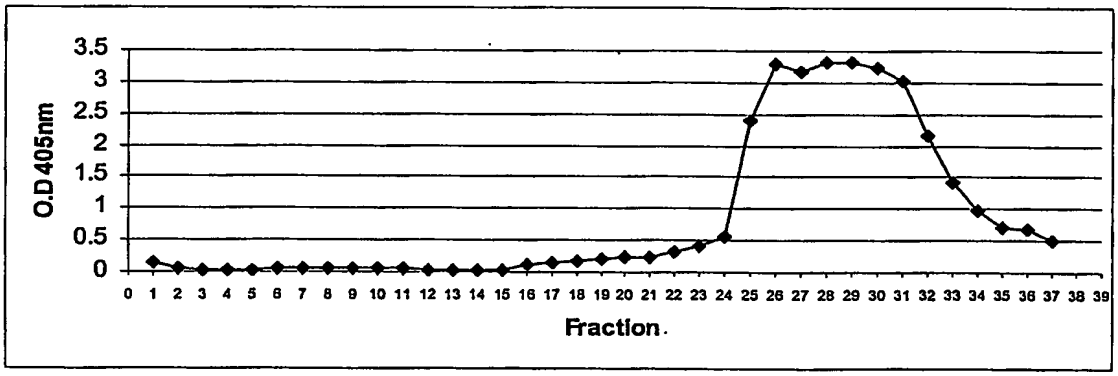

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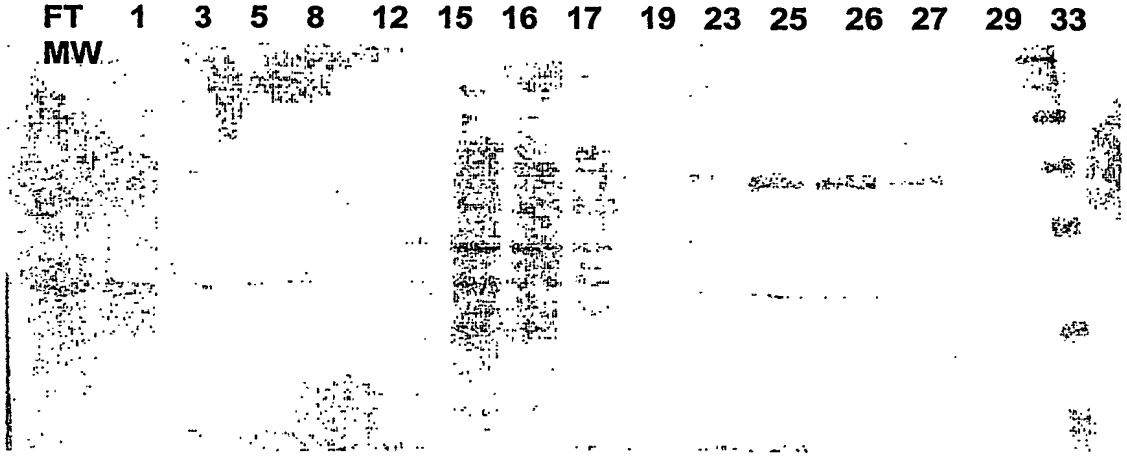

Fig. 3

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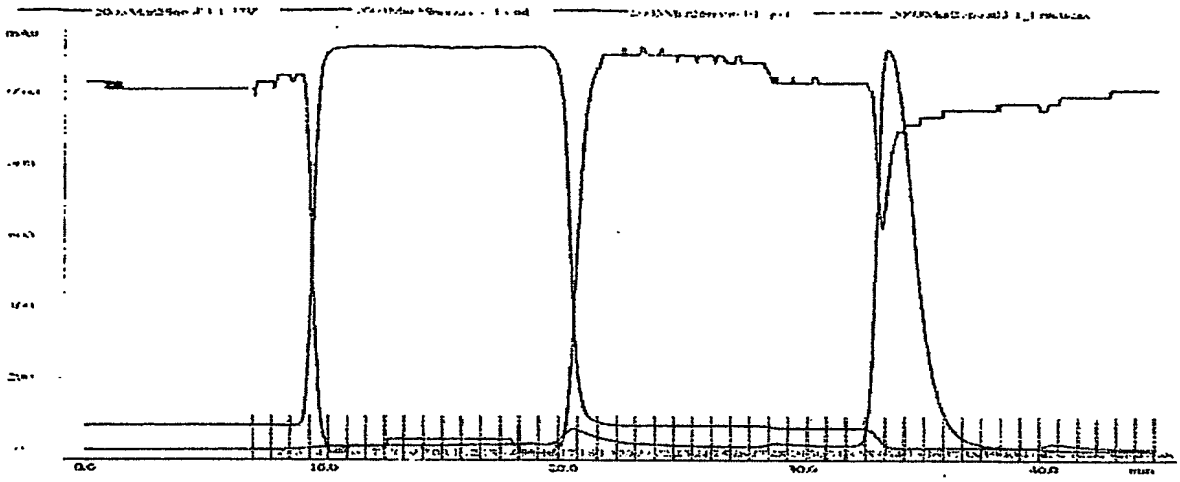
A.



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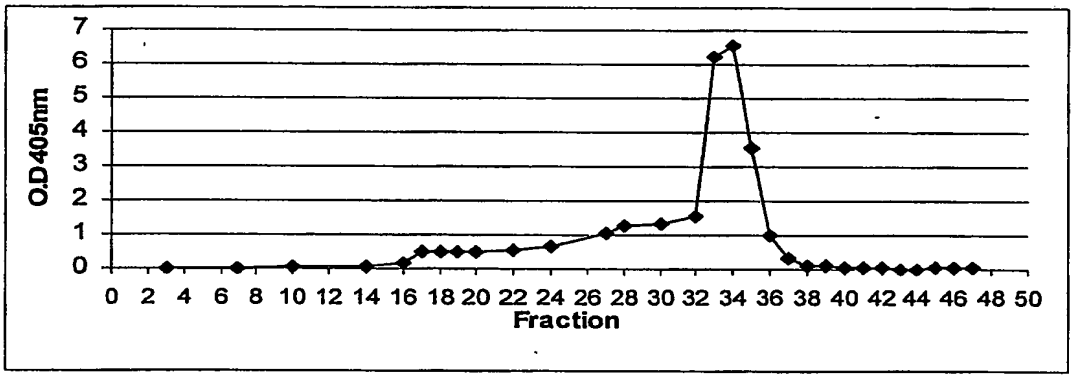

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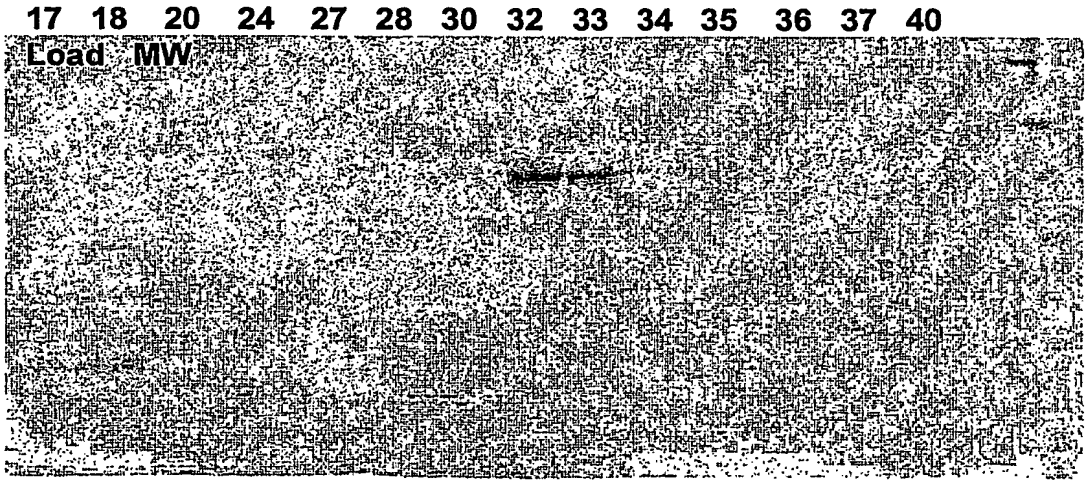

Fig. 4

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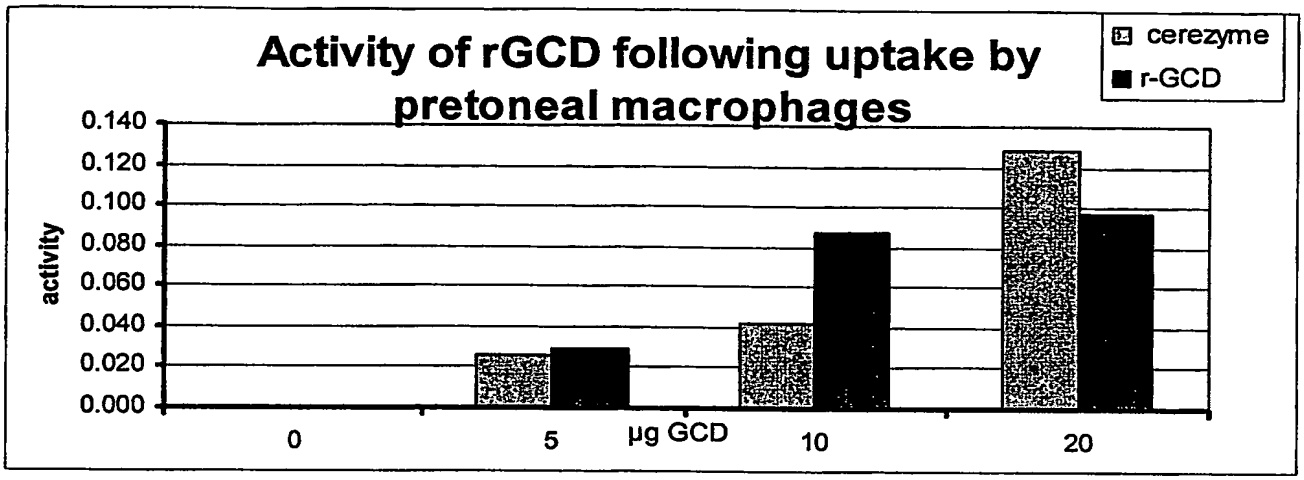

Fig. 5

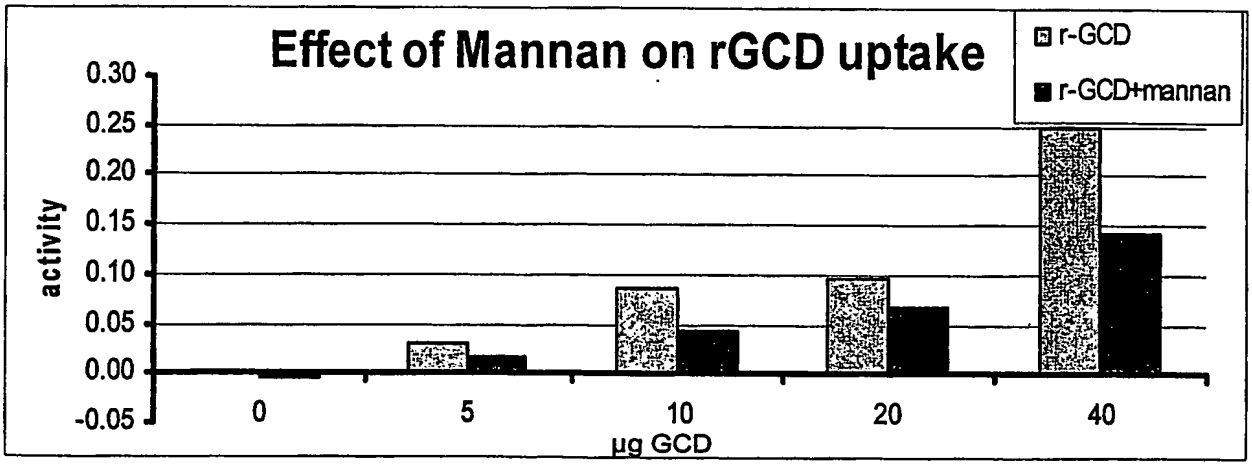

Fig. 6

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